

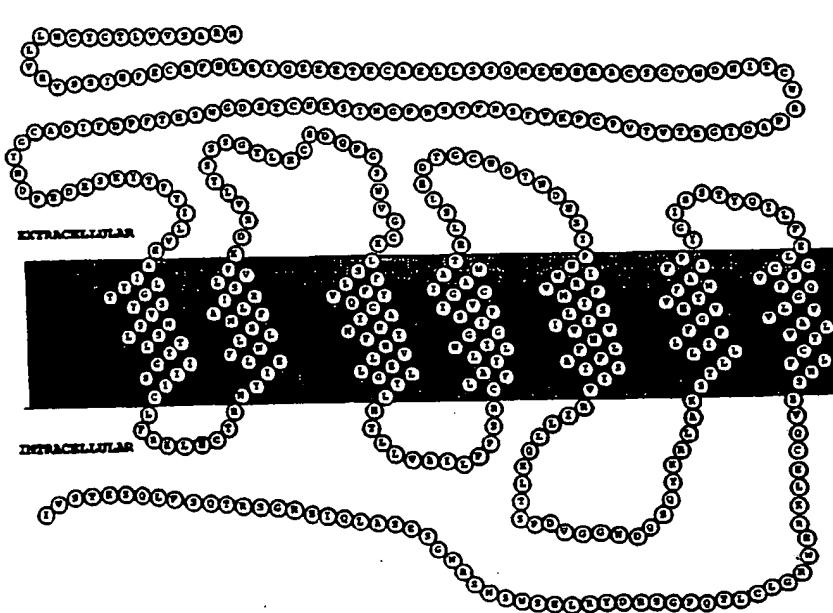
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83/1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :		A1	(11) International Publication Number: WO 95/06724 (43) International Publication Date: 9 March 1995 (09.03.95)
C12N 15/12, C07K 14/705, 16/28, C12N 5/10, G01N 33/68, C12Q 1/68			
(21) International Application Number: PCT/GB94/01892		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).	
(22) International Filing Date: 1 September 1994 (01.09.94)			
(30) Priority Data: 9318105.5 1 September 1993 (01.09.93) GB			
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(54) Title: VIP ₂ (VASOACTIVE INTESTINAL POLYPEPTIDE) RECEPTOR			
			
(57) Abstract			
The present invention provides a VIP ₂ receptor gene (seq ID No.1), and polynucleotide probes specifically binding to this gene or to naturally occurring variants thereof, as well as polypeptide probes specifically binding to the receptor polypeptide or to naturally occurring variants thereof. Expression of the gene in a host is also useful for providing means for use in the evaluation of VIP ₂ agonists and antagonists.			

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0 VIP2 (VASOACTIVE INTESTINAL POLYPEPTIDE) RECEPTOR

Background of invention

Field of invention

This invention relates to the discovery of cDNA sequences encoding for a newly found member of the recently discovered 5 secretin family of G-protein linked neuropeptide-receptors, which is a receptor for vasoactive intestinal polypeptide (VIP), and the application of this discovery to inter alia 1) the development and use of nucleotide sequences derived from the receptor for the measurement (qualitative and 10 quantitative) and regulation of gene expression and 2) the development and use of VIP agonist and antagonist analogues for clinical and therapeutic use in the management of human pituitary dysfunction, various cancers of the brain, pituitary, lung, adrenal, and reproductive system and VIP 15 related disorders of brain function, and other conditions involving VIP.

Description of the Prior art

Vasoactive intestinal polypeptide (VIP) has been identified and purified from small intestine on the basis of its potent 20 vasodilator activity[1, 2]. VIP has a number of actions in the periphery including vasodilatation, stimulation of electrolyte secretion and smooth muscle relaxation[3]. VIP is a member of a family of structurally related polypeptide hormones which include glucagon, glucagon-like peptide I 25 (GLP), peptide histidine isoleucine (PHI), secretin, pituitary adenylate cyclase activating polypeptide (PACAP) and growth hormone releasing hormone (GHRH). All of these peptides are thought to exert their actions through G- protein linked membrane receptors coupled to adenylate 30 cyclase. Recently, a receptor for VIP[4], has been cloned from rat lung[5] and from a human colon carcinoma cell line[4]. Receptors for glucagon[6-8], GLP[9], GHRH[10-12], secretin[13] and PACAP[14-16] have also been cloned.

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Together with receptors for calcitonin[17, 18] and parathyroid hormone (PTH)[19, 20] they form a protein family distinct from other G protein-linked receptors.

VIP has been shown to bind with high affinity to VIP 5 receptors in a variety of tissues. These binding sites are present in lung, liver and intestine, as well as several regions of the brain (e.g. cerebral cortex, hypothalamus and hippocampus[21, 22]). This receptor is probably identical to a receptor known as the PACAP Type II receptor[23, 24] which 10 recognises VIP, PACAP-27 and PACAP-38 with very similar affinities and may correspond to the previously cloned VIP receptor[4, 5] which has a similar tissue distribution[5]. This receptor is present in a variety of peripheral tissues including rat liver, rat lung, mouse splenocytes and human 15 small intestinal epithelium[23, 25-28]. There is some evidence for the existence of other types of VIP receptor in the brain and periphery[29]. Cross-linking studies have been used to identify VIP binding proteins in tissues with molecular weights ranging from 46,000 to 73,000 depending on 20 the tissue and species[30]. Studies with peptide analogs also suggest the existence of more than one pharmacologically distinct class of VIP receptor[31-35]. A class of VIP receptor, for which helodermin is the most potent ligand, has been identified in certain human cell 25 lines, including the lymphoblastic cell line SUP-T1[36, 37] the THP-1 monocyte/macrophage cell line[38] and NCI-H345 lung carcinoma cells[39]. Hill et al. [40] have distinguished two subtypes (or different functional states of a single subtype) of VIP receptor which differ in their 30 sensitivity to GTP analogs. In some brain regions, guanylyl-imidodiphosphate (GMPPNP) substantially inhibited VIP binding. In other regions, VIP binding was insensitive to GMPPNP. Both types of receptor are present in the mouse embryo[41], where they are differentially regulated by 35 treatment with a VIP antagonist.

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To date, however, there have been no reports of the sequencing of any gene encoding a second VIP receptor with a distinct and different molecular structure.

The initial step in VIP action centres on the binding of VIP 5 to a specific membrane bound receptor. Multiple steps are then involved in triggering the synthesis of intracellular cAMP (cyclic Adenosine Monophosphate) in tissues such as brain, pituitary, lung, pancreas, gastrointestinal tract, kidney, reproductive tract, blood vessels and various others 10 and in certain tumours. In the central nervous system, VIP may play a role in the pathogenesis of psychiatric, neurological and neuroendocrine disorders. VIP may also regulate cerebral energy metabolism[42] and neuronal survival[43]. VIP stimulates prolactin secretion from the 15 pituitary[44], catecholamine release from the adrenal medulla[45] and in the immune system it inhibits mitogen activated proliferation of T cells by inhibiting interleukin-2 production[46].

Knowledge of the structure and characteristics of VIP 20 receptors in the brain and pituitary and other tissues would also be helpful both in attempting to use VIP or its analogs in pharmacotherapy and in attempting to understand the possible roles of VIP in the body.

Thus there is a particular need for providing probes and 25 other assay materials and methods for use in the detection and/or identification, directly or indirectly, of abnormalities in VIP receptors, especially human VIP receptors and/or their expression, and/or the genes encoding them, as well as means for use in the evaluation of 30 potential VIP agonists or antagonists for use in the treatment of such abnormalities.

It is an object of the present invention to avoid or minimise one or more of the above problems or disadvantages.

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Summary of Invention

We have now succeeded in the cloning and expression of a specific, adenylate cyclase-linked VIP receptor from rat brain. We have named this receptor the VIP₂ receptor to 5 distinguish it from the receptors cloned from rat by Ishihara et. al. [5] and from human by Sreedharan et. al. [4]. The latter two receptors probably represent species variants of one gene, which we will refer to as the VIP₁ receptor. The VIP₂ receptor was identified by PCR of rat 10 pituitary cDNA using degenerate oligonucleotide primers corresponding to the third and seventh transmembrane domains of the secretin family of G-protein linked receptors. Full length cDNAs were isolated from an olfactory bulb cDNA library. The cDNA sequence for the novel VIP receptor is 15 presented as the basis of this invention. The sequence is presented in interleaved format (see Fig. 1), and has been submitted to the EMBL/Gen Bank database under accession No. Z25885.

Thus we have shown for the first time that VIP receptor 20 occurs naturally in at least two different forms with distinct tissue distributions and different molecular structures. This indicates the possibility of different physiological functions for the different receptors and the need for diagnostic means and methods for distinguishing 25 these different forms, as well as the possibility of providing VIP₂ agonists and antagonists adapted for selective interaction with the newly discovered VIP₂ receptor, for example for the purposes of controlling blood pressure and/or renal function, as well as certain tumours. 30 Given the co-existence of both VIP₁ and VIP₂, provision by means of the present invention of cloned VIP₂ receptor gene and systems for expression thereof, is particularly valuable in providing means for assaying VIP₂ - specific agonists and antagonists.

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In more detail, the present invention provides: a gene encoding VIP₂ receptor; and preferably a gene encoding VIP₂ receptor having substantially the amino acid SEQ ID No:1 disclosed herewith and includes genes encoding VIP receptor 5 which genes have substantial nucleotide sequence homology with the nucleotide sequence SEQ ID No:1 disclosed herein; and in particular the genes of the present invention in a form substantially free from other genes. It will be understood that the genes of the present invention may 10 include nucleic acid sequences (upstream and/or downstream of the receptor coding sequence) which are utilized in the expression of the gene such as promoter, operator, and terminator sequences as well as other sequences which do not inhibit its expression. Thus the expression "gene" includes 15 DNA (including cDNA) and/or RNA sequences as well as plasmid or viral "genes" containing the receptor gene and expression vectors for the gene.

With respect to the distribution of VIP₂ receptor in the body we have found that significant, and in some cases 20 particularly high, concentrations are to be found in inter alia: the supra-chiasmatic nucleus of the brain which is involved in the control of circadian (or diurnal) and other biorhythms including those involved with sleep and arousal, feeding, drinking, various endocrine functions, and 25 ovulation; the dorsal horn of the spinal cord and especially the substantia gelatinosa and Clark's column which are involved in the transmission and processing of pain; and tumours, especially gastro-intestinal and lung tumours.

In one aspect therefore the present invention provides new 30 methods and means based upon the newly discovered VIP₂ receptor nucleotide sequence for use in the clinical diagnosis and therapeutic management of those processes and abnormalities thereof that involve the action of VIP and the VIP₂ receptor, including but not exclusive of:

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Neurological and psychiatric disorders including schizophrenia, depression, Alzheimer's disease and Parkinson's disease; Brain tumours; Pituitary tumours and neuroendocrine disorders; Cardiovascular and inflammatory disorders; Disorders of fertility, control of fertility, testicular tumours; impotence directly related to failure to achieve erection; Disorders of the kidney; Phaeochromocytoma and other tumours of neuroendocrine origin; Lung tumours; Leukaemia, immune and inflammatory disorders; Gastro-intestinal Tumours; Pancreatic tumours, pancreatitis, cystic fibrosis; Diabetes; Disorders of foetal development; especially foetal brain development; and Degenerative disorders of the nervous system.

In addition the invention provides methods and means for use in the control and/or regulation of circadian (or diurnal) and other biorhythms including those involved with sleep and arousal, feeding, drinking, various endocrine functions, and ovulation; as well as of pain thereby providing valuable new alternative means for providing analgesia.

20 In the case of tumours there may be used anti-sense polynucleotides in order to restrict or inhibit the growth of tumours. Preferably there are used anti-sense polynucleotides capable of binding to part of the gene which includes the ATG (initiation) codon corresponding to the 25 start of the VIP2 protein coding region.

In general the VIP2 receptor gene sequences are also useful for the design of oligonucleotide probes capable of specifically hybridising with the genes of the present invention, and for the synthesis of polypeptides which may 30 be used in immunoassays. In addition parts of the cDNA sequence may be used to design and/or provide oligonucleotide probes for use in identifying human and other mammalian VIP receptor genes. Thus the present invention also specifically extends to a human gene DNA

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sequence which has been obtained directly or indirectly through recovery of a human gene by hybridisation thereof with a polynucleotide probe comprising a labelled DNA or RNA sequence capable of specifically hybridizing to a gene 5 encoding VIP2 receptor having the amino acid sequence of SEQ ID No. 1 disclosed herein. In general any part of the VIP2 receptor coding region of the gene may be used for this probe. Both oligonucleotide probes and the polypeptides may be useful for the diagnosis of VIP2 receptor abnormalities. 10 (It will be understood that references to oligonucleotide probes and the use thereof also include such probes as part of longer sequences). Polypeptides encoded within the cDNA sequences may also be used to raise antibodies against selected regions of normal or abnormal VIP2 receptor 15 polypeptide, which are particularly implicated in ligand binding i.e. the first, second, third and fourth extracellular domains of the VIP2 polypeptide (see fig 2); or in signal transduction i.e. the first, second, third and fourth intra-cellular domains, especially the main 20 cytoplasmic loop or third intracellular domain, most preferably in the region of the interface between the third intracellular domain and the sixth transmembrane region. e.g. one of the four extra-cellular domains identified hereinbelow, and for the purification of antibodies directed 25 against such regions. These antibodies may be useful in immunoassays for detecting normal or abnormal VIP₂ receptor in individuals.

Furthermore the invention provides screening means for use in the evaluation of new VIP₂ receptor agonists and 30 antagonists, comprising a cell transformed with a recombinant expression system comprising an open reading frame (ORF) of DNA derived from a VIP₂ receptor gene or VIP₂ receptor cDNA, said ORF being operably linked to a control sequence compatible with said cell, as well as such 35 expression systems per se.

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Thus the present invention further includes a method of producing receptor which method includes the step of expressing the genes of the present invention in a host, as well as VIP₂ receptor produced by such a method. Various 5 suitable hosts are known in the art though eukaryotic hosts are generally preferred, e.g. Xenopus oocytes and COS-7 cells. Prokaryotic hosts that may be used include E. coli. and B. Subtilis. Fungi e.g. yeast may also be used. For the purposes of developing VIP₂ agonist and antagonist 10 analogues, it is particularly preferred to use cloned cell lines expressing human VIP₂ receptor. Genes for human VIP₂ receptor can be isolated and sequenced by screening a human cDNA library preferably a human tumour (e.g. adrenal, pancreas, lung or brain) or normal adult or foetal human 15 brain, with the aid of oligonucleotide probes from the entire rat VIP2 receptor sequence or parts thereof.

Thus the present invention also includes products and processes utilizing, directly or indirectly, human VIP₂ receptor sequences obtained in this way.

20 In particular the invention provides means for the evaluation of new VIP₂ receptor agonists and antagonists. These may employ expression systems of the invention directly or in some cases simply the membranes (with VIP₂ receptor) thereof. Conveniently the transfected or 25 transformed cells, or the membranes thereof, are exposed to a suitable ligand e.g. labelled VIP, and the potential agonist or antagonist and the effect of the latter on the binding of the ligand is monitored, e.g. by examining the variation of binding level with agonist/antagonist dosage. 30 Alternatively or additionally there may be monitored a natural or artificial intra-cellular messenger system such as cAMP production, inositol phosphate turnover, cell calcium concentration, or expression of an artificial gene containing an enzyme marker producing a colour or light 35 reaction etc.

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A suitable method of restriction enzyme analysis in this invention depends on Restriction Fragment Length Polymorphisms (RFLPs). A sample is taken from any suitable tissue such as blood. DNA is extracted from the cells in any 5 conventional way. It is then digested with an appropriate restriction enzyme e.g. one which cuts in CG-rich sequence. The fragments of different length are separated by gel electrophoresis in any conventional way. A restriction fragment pattern is generated. Probing of the fragments will 10 generally be necessary for clearer detection of the pattern and of the fragment(s) of interest, e.g. a fragment which extends from restriction sites "n" to "n + 2" (where "n" denotes any arbitrary number), seemingly not being restricted at the normal site "n + 1" lying between "n" and 15 "n + 2" due to an abnormality at the "normal" restriction site. Alternatively, a polymorphism might generate restriction enzyme sites and thereby given rise to a plurality of shorter fragments where the normal DNA provides longer ones. Whether it is appropriate to probe for long or 20 short fragments will therefore depend on the circumstances of the polymorphism. In some instances, the probe will extend outside the region designated.

Although the RFLP method is one method of assay, it cannot be ruled out that direct hybridisation of probes to the 25 genomic region will be of interest. Thus suitable biopsy or other samples can be subjected to cloning techniques, to isolate a library of genomic DNA, or PCR of genomic DNA or cDNA. Clones containing the gene can be amplified by Polymerase Chain Reaction (PCR) and probes complementary to 30 the said region used directly on PCR products, which need not be first restricted by enzymes.

It will be appreciated, therefore, that the cDNA of the invention also has uses in assays which are not of the RFLP type. Accordingly, the polynucleotides per se are part of 35 this invention, as 'intermediates' suitable (when labelled)

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for use as probes. Both double-stranded and single-stranded polynucleotides are included as well as sense and anti-sense forms. Suitable polynucleotide probes may be oligonucleotides of from 10 to 50, preferably from 16 to 30 5 nucleotides in length. Shorter probes are unlikely to be sufficiently specific for the sequence of interest. Longer polynucleotide probes of from 100 to 500 nucleotides or more may also be used with longer ones (up to 2000 or more nucleotides) being useful e.g. for chromosomal in-situ 10 hybridisation as further described hereinbelow. Preferably the probes relate to parts of the polynucleotide sequence corresponding to one or more domains, or portions thereof, of the VIP2 polypeptide, which are particularly implicated in ligand binding i.e. the first, second, third and fourth 15 extracellular domains of the VIP2 polypeptide (see Figs. 1 and 2); or in signal transduction i.e. the first, second, third and fourth intra-cellular domains, especially the main cytoplasmic loop or third intra-cellular domain, most preferably in the region of the interface between the third 20 intracellular domain and the sixth transmembrane region. The probe will usually be of DNA or RNA and labelled in any suitable manner e.g. by labelling with an enzyme, radioisotope, fluorescent, luminescent, or chemiluminescent labels or biotinylation.

25 The fragments are probed under any appropriate conventional hybridisation conditions, the fragments being conveniently first transferred to a filter. The complexes thus formed are detected by autoradiography or other detection means appropriate to the particular kind of label used.

30 Abnormalities in the polynucleotide sequence of restriction fragments of the genomic DNA or cDNA coding for VIP₂ receptor which are as small as single-point mutations can also be detected by means of Temperature Gradient Gel Electrophoresis in which a temperature gradient is 35 superimposed, parallel to or transversely of, the electrical

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field in gel electrophoresis. The method is based on the fact that the temperature of denaturation of double stranded (ds) DNA is altered by changes in polynucleotide sequence. Furthermore, partial denaturation of a DNA duplex causes a 5 change in electrophoretic mobility. Further details of this technique are described in the literature by Reisner et al, 1989 (56) and, Birmse et al, 1990 (57).

We have now further found that the chromosomal location for the VIP₂ receptor gene is at 7q36.3 and thus the present 10 invention now also provides further diagnostic means for use in the detection of conditions associated with VIP₂ abnormalities, which comprises one or more of hybridisation of a polynucleotide probe of the invention with the 7q36.3 chromosome region; and examination of the 7q36.3 chromosome 15 region for gross abnormalities. Major defects in the 7q36.3 chromosomal region have been found to be associated with the birth defect holoprosencephaly, especially the type 3 form, and thus the present invention also provides a method of diagnosis for holoprosencephaly comprising the steps of 20 collecting a foetal sample and examining the chromosomal content thereof by one or more of the techniques described hereinbefore so as to detect the presence of defects or irregularities in the 7q36.3 chromosomal region.

The VIP₂ receptor cDNA cloned and sequenced is shown in Fig. 25 1A. Fig. 2 illustrates schematically the predicted transmembrane, intracellular and extracellular domains of the receptor molecule. The transmembrane domains consist of seven stretches of hydrophobic in nature amino acids which span the membrane. The extracellular domain consists of four 30 hydrophilic in nature amino acid stretches which exist exterior to the cell membrane. This region is believed to be important for the recognition of specific ligands. The intracellular domain consists of four hydrophilic stretches of amino acids which are thought to be involved in signal 35 transduction.

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It will be appreciated that abnormality in human VIP₂ receptor and/ or its expression, may be "assayed" in a number of ways. Thus the DNA encoding the VIP₂ receptor may itself be assayed for the presence or absence of abnormalities or 5 the VIP₂ receptor polypeptide may be assayed for such purposes, where this is actually expressed.

The former case generally involves the use of labelled polynucleotide probes to hybridise with DNA within the region coding for VIP₂ receptor polypeptide for the purposes 10 of indicating the presence or absence of particular polynucleotide sequences. In the latter case antibody probes are used to form antigen-antibody complexes with regions of the expressed polypeptide for the purposes of indicating the presence or absence of particular polypeptide sequences. 15 It will be understood that once more or less common or typical abnormalities have been specifically identified e.g. by initially probing with 'normal' polynucleotide and then sequencing, polynucleotide probes can be synthesized or otherwise produced with sequences corresponding to or 20 complementary to the "abnormal" sequences, to allow screening of tissue samples for specific VIP₂ receptor gene abnormalities. In the case of gene abnormalities such as the complete or substantial absence of the VIP₂ receptor gene, then this may be detected by failure to yield any 25 binding with suitable probes or absence of any product from PCR amplification using polynucleotide probes complementary to substantially spaced apart portions of the gene.

In the case of assays of the polypeptide itself, suitable stretches of amino acids based on the cDNA sequence 30 information provided by the present information, may be synthesised on a peptide synthesiser. These peptides would generally have a length of from 10 to 50, preferably 15 to 30, amino acids but could be even shorter or longer. Alternatively the complete VIP₂ receptor polypeptide or 35 fragments thereof may be expressed in a suitable eukaryotic

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or prokaryotic host such as E. Coli using an appropriate vector. Polyclonal antibodies to these peptides may be produced by conventional approaches such as the immunisation of host animals (rabbit, goat etc.) with said peptides, 5 optionally conjugated to a protein carrier such as thyroglobulin, and recovery of the desired antibody material therefrom. Monoclonal antibodies could also be raised using conventional monoclonal antibody-production procedures.

It will also be understood that "assay" may be either 10 qualitative or quantitative (e.g. where detection of under or over-expression of the receptor is required). Further preferred aspects of the invention are indicated in the following patent claims.

Detailed Description

15 Isolation and Sequencing of VIP₂ receptor Gene Preparation of Rat Pituitary cDNA

Anterior pituitary glands from male rats (Cob Wistar, 250g) were removed and total RNA was isolated by the method of Chomczynski and Sacchi[47]. Single stranded cDNA synthesis 20 and PCR were carried out using a commercial kit (Perkin Elmer Cetus). 1 μ g RNA was annealed with 2.5 μ M random hexanucleotide primers by heating to 95°C for five minutes, then cooling to 4°C over fifteen minutes. Single stranded cDNA was synthesised by incubating the oligonucleotide-RNA 25 solution at 42°C for 15 minutes in 20 μ l 10mM Tris-HCl, pH 8.3, containing 50mM KCl, 5mM MgCl₂, 1mM each dNTP, 20 units RNase inhibitor and 50 units reverse transcriptase. The reaction was terminated by heating to 99°C for 5 minutes.

Selective Amplification of Rat Pituitary cDNA

30 PCR was performed using a pair of degenerate 32-mer oligonucleotide primers (Figure 3), corresponding to conserved regions in the third and seventh transmembrane domains of the rat secretin[13], pig calcitonin[18] and

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opossum parathyroid hormone PTH[19] receptor. In more detail, each "primer" comprises a mixture of a large number of oligonucleotides with different permutations of nucleotide at particular positions within the 5 oligonucleotide sequence corresponding to the codons for particular amino acids within the receptor peptide sequence coded for thereby. This is necessary in order to include different codons which code for the same amino acid due to the degeneracy of the genetic code. Alternative nucleotides 10 are indicated above each other. Where it is desired to include the possibility of any one of the A, G, C, and T nucleotides (containing the bases Adenine, Guanine, Cytidine, and Thymidine) then the I nucleotide (with the base Inosine) is used. Sequences containing restriction 15 sites (EcoRI and BamHI, respectively) were added to the ends of the two oligonucleotide primers. Sequences of the two oligonucleotides are shown, aligned with the corresponding amino acid sequences in the rat secretin, pig calcitonin and opossum PTH receptors in Fig. 3.

20 Reactions (100 μ l) contained 30pmol of each primer, 20 μ l reverse transcriptase reaction and 2.5 units AmpliTaq DNA polymerase in 50mM KCl, 10mM Tris-HCl, pH 8.3 and 2mM MgCl₂ 25 cycles of PCR (60s denaturation at 94°C, 60s annealing at 45°C, 60s extension at 60°C) were followed by a further 40 25 cycles (60s denaturation at 94°C, 60s + 6s per cycle annealing and extension at 60°C) followed by 7 minutes at 60°C. PCR products were precipitated with ammonium acetate and ethanol after which one half of each reaction was run on a 1% agarose gel. Five bands containing cDNA sequences 30 having a size corresponding generally to that expected of the cDNA within the receptor gene between the two primers used, and ranging in size from 500bp to 900bp were excised from the gel and purified using the Sephaglas BandPrep kit (Pharmacia) and one quarter was used for a 35 further round of PCR (5 cycles of 60s denaturation at 94°C, 60s annealing at 47°C, 60s extension at 60°C followed by a

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further 40 cycles of 60s denaturation at 94°C, 60s + 6s per cycle annealing and extension at 60°C followed by 7 minutes at 60°C.

Preparation of cDNA clones

5 PCR products were ethanol precipitated, digested with BamHI and EcoRI, separated on a 1% agarose/TAE gel, purified using the Sephaglas BandPrep kit (Pharmacia), ligated into pBluescript SK-Vector (Stratagene) and used to transform competent *E. coli* DS941. The sequences of several of the 10 clones were obtained on both strands (Sequenase 2.0 kit, USB). One of the clones (RPR4) was found to have substantial similarity to rat VIP₁ receptor[4, 5] but was distinct from all the known members of the family.

Tissue Distribution of mRNA (Northern Blotting)

15 Total RNA was isolated from tissues using the guanidinium thiocyanate/caesium chloride method[48]. Approximately 20 μ g of each RNA was separated by electrophoresis on denaturing 1% agarose/formaldehyde gels, transferred to a nitrocellulose membrane (Hybond-C, Amersham) and baked for 20 2h at 80°C. The membrane was then hybridised with the insert from RPR4 that had been labelled with [³²P]dCTP-using random hexanucleotide primers (Pharmacia) and the Klenow fragment of *E. coli* DNA polymerase[49]. Hybridisation was performed overnight in 50% formamide, 25mM KP04, pH7.4, 5xSSC 25 (1xSSC=0.15M NaCl, 0.015 M Na citrate pH7.0), 5x Denhardt's solution, 50 μ g/ml salmon sperm DNA. They were then washed twice for 20 min in 2xSSC/0.1%5DS at 50°C, then twice more in 0.5xSSC/0.1%5DS at 50°C and exposed to Fuji RX film. This Northern blot analysis using RPR4 as a hybridisation probe 30 revealed a mRNA transcript of approximately 3.5 kilobases expressed in the pituitary and regions of the brain, with the highest levels being observed in the olfactory bulb.

Screening Rat Olfactory Bulb cDNA Library

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One of the clones isolated, RPR4, was used accordingly to screen a commercial cDNA library in the Lambda Zap II vector[50] prepared from rat olfactory bulb (Stratagene Catalogue No. 936520).

5 4×10^5 plaque forming units of the library (corresponding to one-fifth of the total number of 2×10^6 primary plaques) were plated onto 20x 140mm plates using *E.coli* XL1-blue as a host strain. Filters containing plaque lifts from these plates were denatured by submerging in 1.5M NaCl, 0.5M NaOH for 2
10 mins, followed by neutralization in 1.5M NaCl, 0.5M TrisHCl pH8.0 for 5 mins, then rinsed in 3xSSC. The filters were then blotted dry on Whatman 3MM filter paper and baked at 80°C for 2 hours. They were then hybridised with the insert from RPR4 labelled using the same method found in the
15 Northern Blot protocol as described hereinabove.
Hybridisation was performed overnight in 50% formamide, 6x SSPE, 5x Denhardt's, 0.5% SDS, 100 μ g/ml salmon sperm DNA at 45°C. Positive plaques were identified, picked, and the phage purified by further rounds of plating and screening.
20 This procedure yielded six positive clones, of which three were analysed further. Positive clones were excised by co-infecting *E.coli* strain XL-1 blue with ExAssist (TM) helper phage (Stratagene) Bluescript SK phagemids (packed as filamentousphage particles) were then used to generate
25 double-stranded plasmids by infecting *E.coli* strain SOLRTM, and plating on L-broth/ampicillin plates to produce colonies. The pBluescript SK-double-stranded plasmid was then recovered using standard techniques[51].

Three different clones were isolated and characterised by
30 restriction mapping and sequencing. All three had sequences corresponding to RPR4 but only one cDNA, RPR4/6.3 containing an insert of 3.3 kb, encoded a complete open reading frame, encoding a protein of 437 amino acids with a predicted molecular weight of 49519 (Figure 2). A 22 amino acid
35 hydrophobic signal sequence is found at the amino-terminal

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end, with a predicted signal cleavage site between Pro²² and Glu²³[52]. A hydropathy plot shows seven hydrophobic, putative membrane-spanning domains (Figure 2). Comparison with other members of the secretin/calcitonin/PTH receptor 5 family (Figure 3) revealed that the predicted protein encoded by RPR4/6.3 has greatest similarity with the rat VIP₁ and PACAP type I receptors (50% identity, with each). The highest amino acid sequence identity is found in the putative transmembrane regions, whereas the sequences of the 10 amino-terminal extracellular domains and the carboxyl-terminal cytoplasmic ends are highly divergent.

Expression of VIP₂ receptor Gene

In order to determine the pharmacological characteristics of the novel receptor, the insert from the full length clone 15 (RPR4/6.3) was excised as an EcoRI fragment and ligated into the EcoRI site of the mammalian cell expression vector pcDNA-1 (Invitrogen)[53, 54], and transiently transfected into COS-7 cells.

COS 7 cells were grown in DMEM supplemented with 10% new- 20 born calf serum and 100U/ml each of penicillin and streptomycin, in a humidified atmosphere of 95% air/ 5% CO₂ at a constant temperature of 37°C, and were passaged every 3-4 days. Cells for transfection were trypsinised the day before the experiment and plated at a density of 25 approximately 40-50% confluence in 75cm² flasks.

For transfection, cells were washed twice with OptiMEM (Gibco) supplemented with 100U/ml each of streptomycin and penicillin at 37°C before exposure to transfecting medium for 4h. The transfecting medium consisted of OptiMEM/ 30 penicillin/ streptomycin, 400μg/ml DEAE dextran (Promega), 100μM chloroquine phosphate (Sigma) and 10-20μg plasmid per flask. This was replaced with 10% DMSO in PBS for 2 min, then DMEM/ 2% UltraSer G/ penicillin/ streptomycin. Cells

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were grown for 24h, then trypsinised and re-plated. Cells were harvested 48h later.

Functional Assay of VIP₂ receptor Gene

Since all members of the secretin receptor family so far 5 identified are associated with activation of adenylate cyclase (also referred to in the art as adenylyl cyclase), the transfected cells were stimulated with several potential ligands, and intracellular cAMP levels measured by radioimmunoassay.

10 For screening purposes, cells transfected with RPR4/6.3 were seeded onto 12-well tissue culture dishes, and for dose/response experiments, onto 24-well dishes. Prior to incubation with various peptide ligands, the cultures were washed with DMEM containing 0.25% BSA, and 15 pre-incubated at 37 C for 30 min in the presence of 0.5mM isobutyl methylxanthine (IBMX). VIP ligand and other peptides were directly added at concentrations indicated in Figs. 4a and 4b and incubated at 37 C for 15-30 min. The reaction was stopped by adding ice cold 0.1M HCl, and the 20 cells homogenised by trituration. The levels of cAMP in the acidic extracts were measured by radioimmunoassay using antiserum cAB4 (courtesy of K.J. Catt, NICHD, NIH, Bethesda, MD) [55].

Results

25 Fig. 4a shows the effect of stimulating cells transfected with RPR4/6.3 by various peptide ligands. Values of cAMP release are expressed (mean \pm SEM, n=3) as a percentage of the stimulation evoked by 100nM VIP. While treatment with 30 corticotropin releasing factor (CRF), calcitonin gene related peptide (CGRP), secretin and glucagon, exhibited negligible effect, treatment with VIP, PACAP27, and PACAP-38 resulted in a marked elevation of cAMP levels. PHI and rat GHRH (rGHRH) also stimulated cAMP levels but were significantly less potent. PHI, VIP and PACAP-38 failed to

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stimulate cAMP levels in a control experiment where COS-7 cells had been transfected with the 5HT_{1A} (Serotonin) receptor (data not shown).

As shown in Fig.4b the stimulation of cAMP levels by VIP, 5 PACAP, helodermin, and PHI was dose-dependent. The EC₅₀ (peptide concentration for 50% of maximal effect) for cAMP accumulation was approximately 0.18nM for PACAP-38, 0.43nM for PACAP-27, 0.25nM for helodermin, 0.17nM for VIP and 2.14nM for PHI. (The values shown in Fig.4b are the mean of 10 3. Basal cAMP was 0.8±0.15 pmoles/well). The maximal stimulation of cAMP accumulation by rGHRH (400nM) was only 60% of that found for VIP. We therefore conclude that the order of potency of these ligands is VIP- PACAP38- PACAP27- helodermin >PHI >>rGHRH, and that accordingly, RPR4/6.3 15 encodes a high affinity receptor for VIP.

Description of the Figures

Fig. 1 shows the gene encoding the rat VIP₂ receptor. The polynucleotide sequences specifically elucidated thus far are indicated along with the deduced amino-acid 20 sequence;

Fig. 2 shows schematically the 7 transmembrane domains of the rat VIP₂ receptor and the 4 extracellular and 4 intracellular domains of the amino acid sequence derived from RPR4/6.3 using standard single letter codes to 25 represent the amino acids;

Fig.3 shows the sequences for the two degenerate oligonucleotide primers; and

Fig.4 shows cAMP accumulation monitored during functional assay of a cloned gene of the invention.

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CLAIMS

1. A gene encoding VIP₂ receptor.
2. A gene encoding VIP₂ receptor having the amino acid sequence of SEQ ID No.1.
3. An isolated DNA sequence encoding VIP₂ receptor.
4. A recombinant DNA sequence encoding VIP₂ receptor.
5. An isolated or recombinant DNA sequence for use in expression in a prokaryotic or eukaryotic host cell of a polypeptide product having an amino acid sequence corresponding to a sufficient extent to that of a naturally occurring VIP₂ receptor to provide at least one biological function of said naturally occurring VIP₂ receptor, which DNA sequence is selected from:
 - a) the DNA sequence of SEQ ID No. 1 or a sequence complementary thereto; and
 - b) a DNA sequence which can hybridize to a DNA sequence defined in (a) or to a fragment thereof.
6. A gene or sequence according to any one of claims 1,3,4 and 5b wherein said VIP₂ receptor is human VIP₂ receptor.
7. A gene or sequence according to claim 6 which has been obtained directly or indirectly through recovery of a human gene by hybridisation thereof with a polynucleotide probe comprising a labelled DNA or RNA sequence capable of specifically hybridizing to a gene according to claim 2.
8. A prokaryotic or eukaryotic host cell transformed or transfected with a gene or DNA sequence according to any one of the preceding claims.

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9. A recombinant vector containing a DNA sequence or gene according to any one of claims 1 to 7.

10. A prokaryotic or eukaryotic host cell transformed or transfected with a recombinant vector according to claim 9.

11. A polynucleotide probe comprising a labelled DNA or RNA sequence capable of specifically hybridizing to a gene according to claim 1, claim 2 or claim 6 when dependent on claim 1 or claim 2, or a naturally occurring variant thereof.

10 12. A method of detecting a gene according to claim 1 or claim 2 or claim 6 when dependent on claim 1 or claim 2, which method comprises hybridizing a probe according to claim 11 with said gene, and detecting bound labelled probe.

13. An antibody probe comprising a labelled antibody raised 15 against an amino acid sequence capable of specifically binding to VIP₂ receptor expressed by a gene according to claim 1 or claim 2 or claim 6 when dependent on claim 1 or claim 2, or a naturally occurring variant thereof.

14. An antibody probe according to claim 13 which is a 20 monoclonal antibody.

15. A method of detecting VIP₂ receptor expressed by a gene according to claim 1 or claim 2 or claim 6 when dependent on claim 1 or claim 2, or a naturally occurring variant thereof, which method comprises allowing a probe according 25 to claim 13 or claim 14 to bind with said receptor, and detecting bound labelled probe.

16. A method of diagnosis for a condition associated with VIP₂ receptor abnormality which comprises hybridizing a polynucleotide probe according to claim 11 with the 7q36.3

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chromosomal region, and monitoring for absence of bound labelled probe.

17. A method according to claim 16 wherein is used the technique of fluorescence *in situ* hybridisation.

5 18. A method of diagnosis for a condition associated with VIP₂ receptor abnormality which comprises amplification by polymerase chain reaction using polynucleotide primers from substantially spaced apart portions of a nucleic acid sequence encoding VIP₂ receptor, and monitoring for absence 10 of amplified polynucleotide sequence product.

19. A method of diagnosis for a condition associated with VIP₂ receptor abnormality which comprises microscopic examination of the chromosomal region for gross abnormality.

20. A method of diagnosis for holoprosencephaly comprising 15 the steps of collecting a foetal sample and examining the chromosomal content thereof by a method according to any one of claims 16 to 19.

21. A method of evaluating a potential VIP₂ agonist or antagonist comprising the steps of: providing cell membrane 20 of a VIP₂ receptor expression system comprising a transformed or transfected host cell according to claim 8 or claim 10, which membrane has VIP₂ receptor; exposing said cell membrane to labelled ligand and to said agonist or antagonist; and monitoring at least one of ligand binding 25 and an intra-cellular messenger system.

22. A method according to claim 21 which includes the preliminary step of providing a VIP₂ receptor expression system comprising a transformed or transfected host cell according to claim 8 or claim 10.

1/6FIG. 1

SEQ ID NO: 1
 SEQUENCE TYPE: Nucleotide with
 corresponding protein
 SEQUENCE LENGTH: 2126 base
 pairs

STRANDEDNESS: double
 TOPOLOGY: linear
 MOLECULE TYPE: cDNA TO mRNA

ORIGINAL SOURCE
 ORGANISM: rat
 IMMEDIATE EXPERIMENTAL
 SOURCE
 Olfactory bulb

FEATURES
 from 69 to 134 bp signal peptide
 from 135 to 1382 bp mature peptide
 from 237 to 239 bp potential
 glycosylation site
 from 327 to 329 bp potential
 glycosylation site
 from 339 to 341 bp potential
 glycosylation site
 from 447 to 512 bp transmembrane
 domain 1 (putative)
 from 540 to 599 bp transmembrane
 domain 2 (putative)
 from 675 to 746 bp transmembrane
 domain 3 (putative)
 from 783 to 851 bp transmembrane
 domain 4 (putative)
 from 903 to 974 bp transmembrane
 domain 5
 from 1050 to 1109 bp transmembrane
 domain 6 (putative)
 from 1146 to 1205 bp transmembrane
 domain 7 (putative)

PROPERTIES: VIP2 receptor for
 vasoactive intestinal peptide
 (VIP)

GCGCTGGGAG	GCCCCGAGCT	GGCGTTACTG	CTGAGGGCGC	CAAGGACCGA	GGCGGGCACTG	60												
AGCCCAGG	ATG	AGG	GCG	TG	GTC	CTG	ACC	TGC	TAC	TGC	TGG	TTG	CTG	GTG	113			
Met	Arg	Ala	Ser	Val	Val	Leu	Thr	Cys	Tyr	Cys	Trp	Leu	Leu	Val				
-20														-10				
CGG	GTG	AGC	AGC	ATC	CAC	CCA	GAA	TGC	CGG	TTT	CAT	CTG	GAA	ATA	CAG	GAA	164	
Arg	Val	Ser	Ser	Ile	His	Pro	Glu	Cys	Arg	Pro	Phe	His	Leu	Glu	Ile	Gln	Glu	
-5															10			
GAG	GAG	ACA	AAA	TGT	GCA	GAG	CTG	CTA	AGC	AGC	CAA	ATG	GAG	AAT	CAC	AGA	215	
Glu	Glu	Thr	Lys	Cys	Ala	Glu	Leu	Leu	Ser	Ser	Gln	Met	Glu	Asn	His	Arg		
15															25			
GCT	TGC	AGC	GGT	GTC	TGG	GAC	AAC	ATC	ACA	TGC	TGG	CGC	CCT	GCA	GAC	ATT	266	
Ala	Cys	Ser	Gly	Val	Trp	Asp	Asn	Ile	Thr	Cys	Trp	Arg	Pro	Ala	Asp	Ile		
30															40			

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GGG GAA ACT GTC ACA GTG CCC TGC CCC AAA GTG TTC AGC AAT TTC TAC AGC Gly Glu Thr Val Thr Val Pro Cys Pro Lys Val Phe Ser Asn Phe Tyr Ser 45 50 55 60	317
AGA CCA GGA AAC ATA AGC AAA AAC TGC ACT AGT GAT GGG TGG TCG GAG ACA Arg Pro Gly Asn Ile Ser Lys Asn Cys Thr Ser Asp Gly Trp Ser Glu Thr 65 70 75	368
TTT CCG GAT TTC ATA GAT GCG TGT GGC TAC AAC GAC CCC GAG GAT GAG AGT Phe Pro Asp Phe Ile Asp Ala Cys Gly Tyr Asn Asp Pro Glu Asp Glu Ser 80 85 90 95	419
AAG ATC ACG TTT TAT ATT CTG GTG AAG GCC ATT TAT ACC TTG GGC TAC AGT Lys Ile Thr Phe Tyr Ile Leu Val Lys Ala Ile Tyr Thr Leu Gly Tyr Ser 100 105 110	470
GTT TCT CTG ATG TCT CTT ACA ACA GGA AGC ATA ATT ATC TGC CTC TTC AGG Val Ser Leu Met Ser Leu Thr Thr Gly Ser Ile Ile Cys Leu Phe Arg 115 120 125	521
AAG CTG CAC TGC ACA AGG AAC TAC ATC CAC CTG AAT CTG TTC CTC TCC TTC Lys Leu His Cys Thr Arg Asn Tyr Ile His Leu Asn Leu Phe Leu Ser Phe 130 135 140 145	572
ATG CTG AGA GCC ATC TCT GTG CTG GTC AAG GAC AGT GTG CTC TAC TCC AGC Met Leu Arg Ala Ile Ser Val Leu Val Lys Asp Ser Val Leu Tyr Ser Ser 150 155 160	623
TCA GGT ACA CTG CGC TGC CAC GAC CAG CCG GGC TCC TGG GTT GGC TGC AAG Ser Gly Thr Leu Arg Cys His Asp Gln Pro Gly Ser Trp Val Gly Cys Lys 165 170 175 180	674
CTC AGC CTG GTA TTC TTC CAG TAC TGT ATC ATG GCG AAC TTC TAC TGG CTT Leu Ser Leu Val Phe Phe Gln Tyr Cys Ile Met Ala Asn Phe Tyr Trp Leu 185 190 195	725
CTG GTG GAG GGT CTC TAC CTG CAC ACC CTC CTG GTA GCC ATC CTT CCT CCC Leu Val Glu Gly Leu Tyr Leu His Thr Leu Leu Val Ala Ile Leu Pro Pro 200 205 210	776
AGC AGG TGT TTC CTG GCC TAC CTT CTT ATT GGA TGG GGT ATC CCC AGT GTG Ser Arg Cys Phe Leu Ala Tyr Leu Ile Gly Trp Gly Ile Pro Ser Val 215 220 225 230	827
TGT ATA GGT GCA TGG ATA GCA ACT CGC CTT TCT TTA GAA GAC ACA GGT TGC Cys Ile Gly Ala Trp Ile Ala Thr Arg Leu Ser Leu Glu Asp Thr Gly Cys 235 240 245	878
TGG GAC ACG AAC GAC CAC AGC ATC CCC TGG TGG GTC ATT CCG ATG CCC ATT Trp Asp Thr Asn Asp His Ser Ile Pro Trp Trp Val Ile Arg Met Pro Ile 250 255 260 265	929
CTA ATT TCT ATT GTA GTC AAC TTT GCC CTC TTC ATC AGC ATT GTA AGG ATC Leu Ile Ser Ile Val Val Asn Phe Ala Leu Phe Ile Ser Ile Val Arg Ile 270 275 280	980
TTA CTT CAG AAG CTA ACT TCT CCA GAT GTT GGT GGC AAT GAC CAG TCA CAG Leu Leu Gln Lys Leu Thr Ser Pro Asp Val Gly Gly Asn Asp Gln Ser Gln 285 290 295	1031
TAC AAG AGG CTC GCC AAG TCC ACA CTG CTG CTA ATC CCA CTG TTT GGC GTC Tyr Lys Arg Leu Ala Lys Ser Thr Leu Leu Ile Pro Leu Phe Gly Val 300 305 310 315	1082

CAC TAC ATG GTG TTT GCT GCC TTC CCC ATT GGC ATC RCC TCC ACG TAC CAG His Tyr Met Val Phe Ala Ala Phe Pro Ile Gly Ile Ser Ser Thr Tyr Gln 320 325 330	1133
ATC CTG TTT GAG TTA TGT GTT GGT TCC TTC CAG GGC CTG GTG GTC GCA GTT Ile Leu Phe Glu Leu Cys Val Gly Ser Phe Gln Gly Leu Val Val Ala Val 335 340 345 350	1184
CTA TAT TGC TTT CTG AAC AGT GAG GTA CAG TGT GAA CTG AAA AGA AGG TGG Leu Tyr Cys Phe Leu Asn Ser Glu Val Gln Cys Glu Leu Lys Arg Arg Trp 355 360 365	1235
AGA GGC CTG TGC CTG ACC CAG CCT GGG AGC CGG GAC TAC CGG CTG CAC AGC Arg Gly Leu Cys Leu Thr Gln Pro Gly Ser Arg Asp Tyr Arg Leu His Ser 370 375 380	1286
TGG TCC ATG TCC CGG AAT GGC TCA GAA AGC GCC CTA CAG ATA CAC CGT GGC Trp Ser Met Ser Arg Asn Gly Ser Glu Ser Ala Leu Gln Ile His Arg Gly 385 390 395 400	1337
TCC CGT ACC CAG TCC TTC CTG CAG TCA GAG ACC TCA GTC ATT TAGCTGTGTC Ser Arg Thr Gln Ser Phe Leu Gln Ser Glu Thr Ser Val Ile 405 410	1389
CCTCATACAG AGCTGACAGT GCTGCTGGGT TTGACATATG TGTGCGCAG CTTCTCTGC 1449 TTGCCCCAGT GTCTGGTGCC TTATTGGGTC AGCCCTGGTC CTTAACCTCGA TTGTAACCTG 1509 ATTGAAACAC CAGTTATTGT TGACAGACTC TAGCCTTTAA GCCATCCCTC TCATAATATG 1569 GCACAGCCAT ATTCTACTTT CAAAGAGAGC AAGGAAACCA GGTGGCCCTG AACATCAAAA 1629 CTGGGTCTA GAACGTCGGA AAAAAACAA GGAGGAAGAT TCCAGTTCTC CCACTGCCTC 1689 CTGTCAGGGA GAACCAACGTC CAGAACCCAGC TGAGCATCAC CACCTGTGG CCCAGCGCAT 1749 GCCTATGGTT GTCTCTGACA TTCCCTCTCAA TCTATGGCAT ACCAGGGAAA GTGGATGAAA 1809 GCAACCTTCT CATCCTACAA GCTTCCTGGT GCCCCCGGCC TTGAGTTCTG TCCTGTGGAC 1869 TACATCTGGA CAGCCACACC CTTGCCATTC CCTGGACCAT CTGGTAGTGA CCAAAGATAA 1929 GTCAGGGAGG ATATTCTATGG TTCAGCGGCC AAGCCAGGAA CTCATCTCT CCCAGGGCAC 1989 TTAGCATCTG CTGCTTCCTA CAAGTGGTGA GCAGCCCTGG ATCCCAGGCC GGCTGCTGGC 2049 ATGAAGGCC CTCATTCTATGA CTTGTCCATT CAGCCCAAGG TTGTTCTCTA GCTGGTAGAA 2109 AAACTTCGAT TTTTATT	2126

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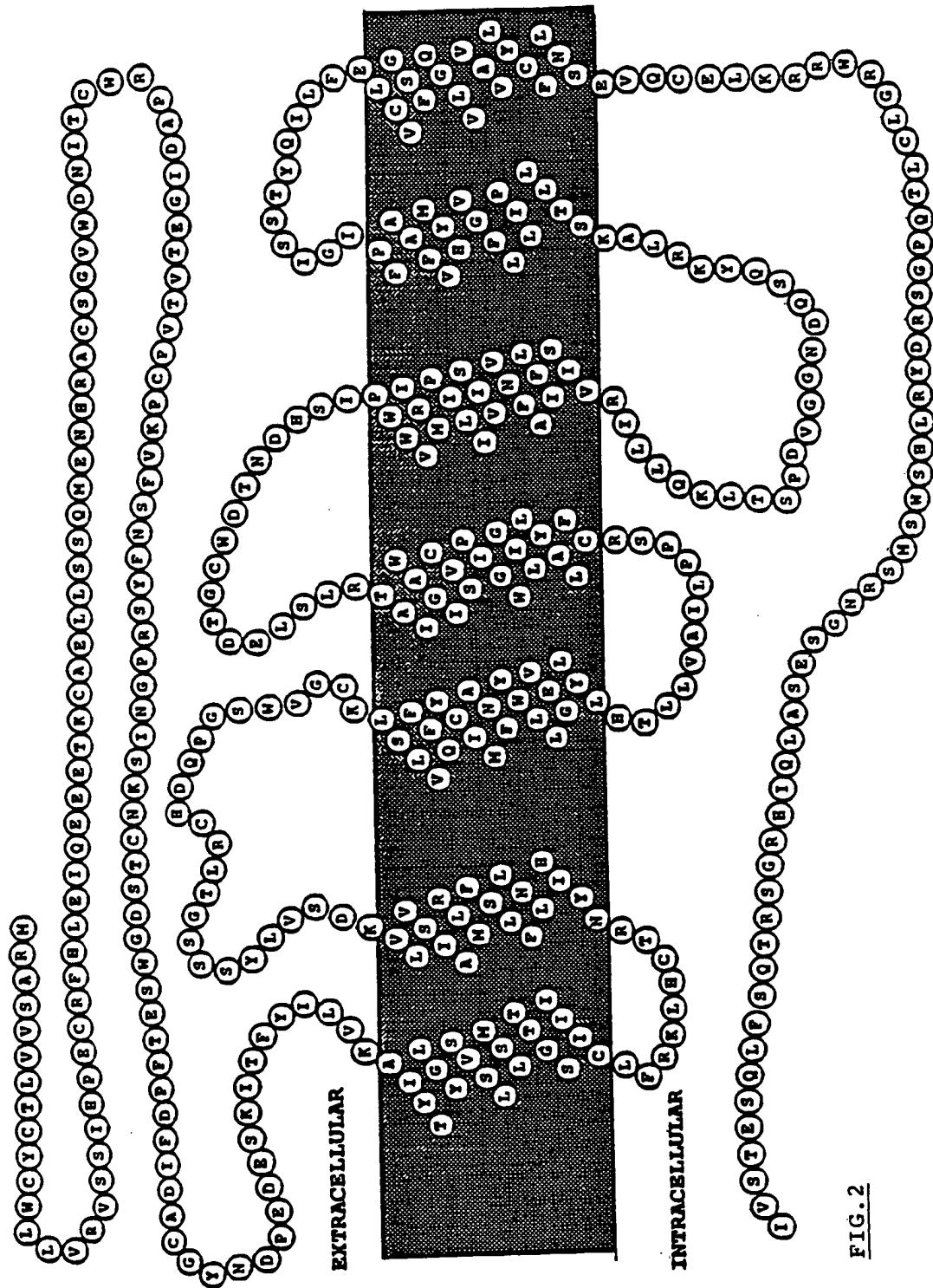


FIG. 2

5/6FIG. 3

Calcitonin	Asn Tyr Phe Trp Met Leu Cys Glu Gly Val Tyr
Parathyroid hormone	Asn Tyr Tyr Trp Ile Leu Val Glu Gly Leu Tyr
Secretin	Asn Tyr Ala Trp Leu Leu Val Glu Gly Leu Tyr

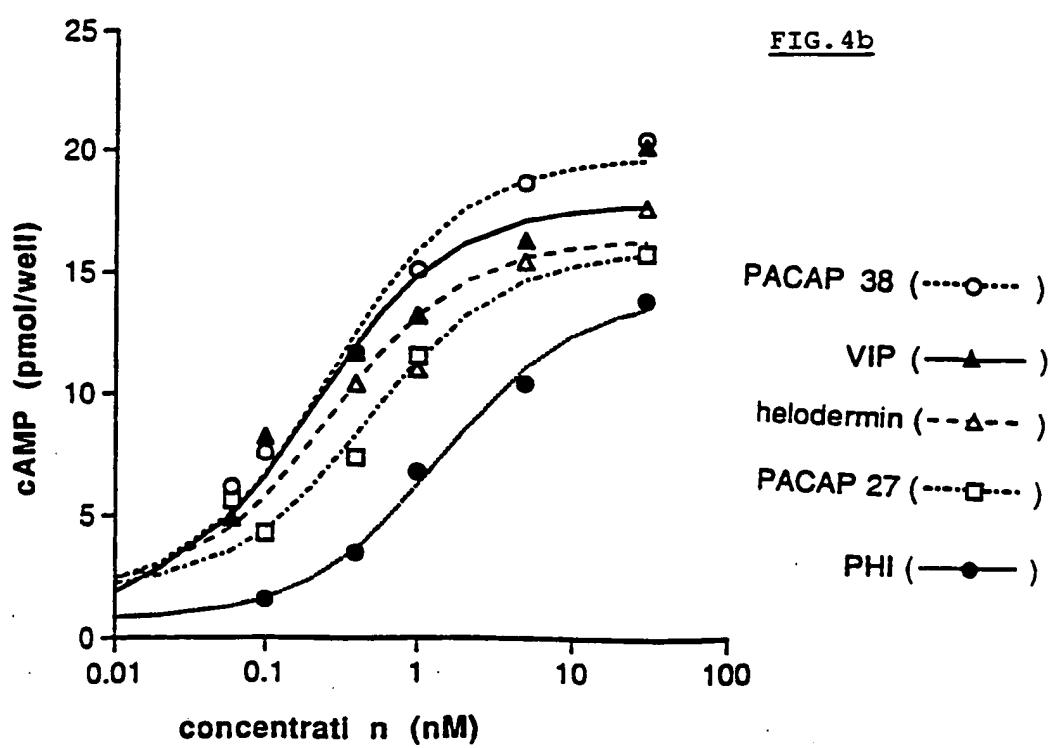
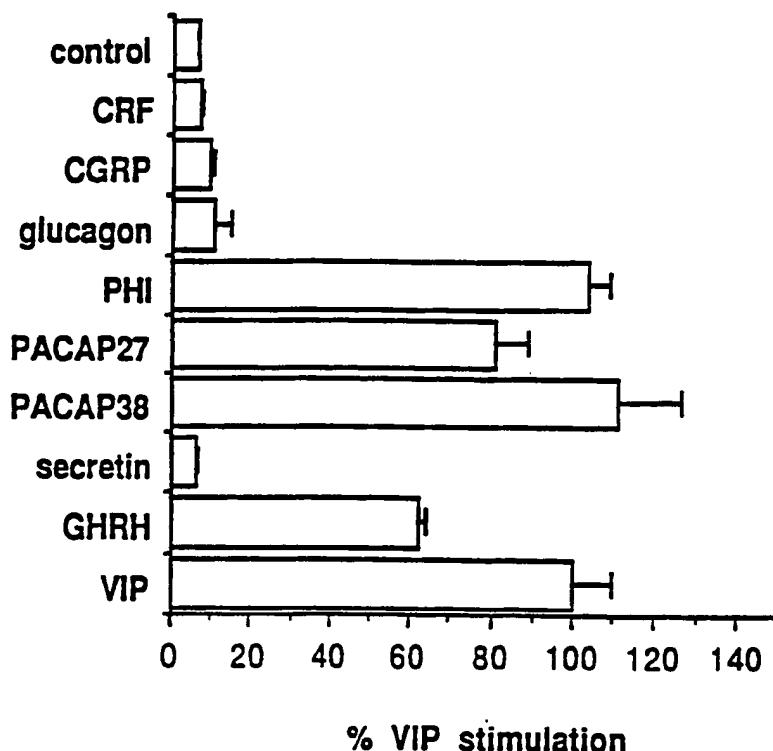
Oligo 1 5'-CCGAATTC ~~AA~~^C ~~T~~^C ~~T~~^A ~~C~~^T ~~T~~^C ~~T~~^A -3'

(Third Transmembrane)

Calcitonin	Phe Gln Gly Phe Phe Val Ala Ile Ile Tyr Cys
Parathyroid hormone	Phe Gln Gly Phe Phe Val Ala Ile Ile Tyr Cys
Secretin	Phe Gln Gly Leu Val Val Ala Val Leu Tyr Cys

Oligo 2 3'- ~~AA~~^A ~~C~~^T~~C~~ ~~C~~^T ~~A~~^A ~~T~~^A ~~C~~^T ~~C~~^T ~~A~~^A ~~T~~^A ~~C~~^T ~~C~~^T ~~A~~^A ~~T~~^A ~~C~~^T ~~C~~^T ~~A~~^A ~~T~~^A ~~C~~^T CCTAGGGG-5'

(Seventh Transmembrane)

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/01892

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6	C12N15/12	C07K14/705	C07K16/28	C12N5/10	G01N33/68
C12Q1/68					

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>FEBS LETTERS., vol.334, no.1, 8 November 1993, AMSTERDAM NL pages 3 - 8 LUTZ EM; SHEWARD WJ; WEST KM; MORROW JA; FINK G; HARMAR AJ; 'The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide.' see the whole document</p> <p>—</p> <p>WO,A,92 21754 (OSAK BIOSCIENCE KENKYUSHO) 10 December 1992 see figure 1</p> <p>—</p> <p style="text-align: right;">-/-</p>	1-22
X		5-12

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *'A' document defining the general state of the art which is not considered to be of particular relevance
- *'E' earlier document but published on or after the international filing date
- *'L' document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *'O' document referring to an oral disclosure, use, exhibition or other means
- *'P' document published prior to the international filing date but later than the priority date claimed

- *'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *'&' document member of the same patent family

1 Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1994	30.12.94
Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nauche, S

INTERNATIONAL SEARCH REPORT

Inte	nal Application No
PCT/GB 94/01892	

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>DATABASE WPI Week 9344, Derwent Publications Ltd., London, GB; AN 93-348480 & JP,A,5 255 394 (ZH OSAKA BIOSCIENCE KENKYUSHO) 5 October 1993 see abstract</p> <p>-----</p>	5-12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 94/01892

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9221754	10-12-92	JP-A- 4360688	14-12-92

Form PCT/ISA/210 (patent family annex) (July 1992)